Review Article

SPOP promotes CDCA5 degradation to regulate prostate cancer progression via the AKT pathway

Zhenzhen Luo [a]; Jing Wang [a]; Yue Zhu [a]; Xiao Sun [a]; Chenchen He [a]; Mengjiao Cai [a]; Jinlu Ma [a]; Yi Wang [b,*]; Suxia Han [a,**]

[a] Department of Oncology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China
[b] State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Center for Protein Sciences (Beijing), Beijing Institute of Lifeomics, Beijing, China

Abstract

The E3 ubiquitin ligase adaptor Speckle-type POZ protein (SPOP) plays an important tumour suppressor role in prostate cancers (PCa), with mutation rate up to 15%. However, how SPOP mutations regulate prostate tumorigenesis remains elusive. Here, we report the identification of cell division cycle associated 5 (CDCA5) as a SPOP substrate. We found that SPOP interacts with CDCA5 and promotes its polyubiquitin degradation in a degron-dependent manner. This effect was greatly impaired by introducing PCa associated SPOP mutations. Importantly, we found that CDCA5 was essential for PCa cells to survive and proliferate. CDCA5 depletion in PCa cells led to cessation of proliferation, G2M arrest, severe sister chromatid aggregation disturbance, and apoptosis. we also found that CDCA5 knockdown decreased the protein expression of p-GSK3β, increased the activity of caspase-3, caspase-9, and the Bax/Bcl-2 ratio. Besides, we confirmed that CDCA5 interrupted cancer cell behavior via the AKT pathway. In contrast, silencing SPOP or overexpressing CDCA5 increased cell proliferation. Consistently, depleting SPOP along with CDCA5, or overexpressing CDCA5 along with SPOP also caused the growth of cells repressed. Consistent with the functional role of CDCA5, the mRNA and protein levels of CDCA5 were significantly increased in PCa, compared to normal tissues, and its high expression was associated with more severe lymph node metastasis, higher Gleason score, and poorer prognosis. Together, our data showed that SPOP plays a crucial role in inhibiting tumorigenesis and partly achieved this by promoting the degradation of oncoprotein CDCA5.

Introduction

As a cullin-3- based ubiquitin (Ub) ligase adaptor, Speckle-type POZ protein (SPOP) structurally comprises a N-terminal MATH domain that recognizes substrates, and a C-terminal BTB/POZ domain that binds the Cullin3-Ring ligases scaffold protein [1]. SPOP gene mutations impair its substrate binding ability and are highly relevant to human prostate cancers (PCa), with a mutation rate of 10% to 15%. The frequently mutated sites often occur in MATH domain, including Y87, F102, F125, K129, D130, W131, and F133 [2–4]. SPOP exerts a tumor suppressor effect in many tumors including PCa by targeting oncoprotein substrates, such as AR [5], ERG [6], TRIM24 [7], NANOG [8], CDC20 [9], and others [10-12]. Meanwhile, it has been reported that SPOP is overexpressed in nearly 100% of clear cell renal cancer tumor tissues (RCC) and displays a possible oncogenic property [13]. The physiological role of SPOP in the occurrence and development of cancer is far from clarified. Therefore, a better understanding of the regulatory network of SPOP affecting the PCa is very important for both mechanistic studies and cancer treatment.

Cell Division Cycle Associated 5 (CDCA5), also known as Sororin (origins in Latin Soror, meaning sister), serves as a regulator for the aggregation and separation of sister chromatids to ensure the accurate distribution of chromosomes in meiotic and mitotic cells [14, 15]. CDCA5 depletion leads to mitotic arrest and complete loss of sister chromatid cohesion [16]. Although CDCA5 expression can be regulated at both transcription and post-translational modification levels under physiological conditions, how it is regulated under pathological conditions remains unclear,

because cancer cells are insensitive to growth-inhibiting signals with disrupted cell cycle [17]. CDCA5 has been implicated in the pathology of a variety of tumors and is associated with tumor relapse and poor prognosis among several types of cancers, including liver, lung, esophageal, and colorectal cancer [18-21].

In this study, we investigated the physiological role of SPOP in regulating tumorigenesis. To this end, identified oncoprotein CDCA5 as a new ubiquitin substrate of SPOP in PCa through a proteomic assay and validated the findings by biochemical and functional assays. We also investigated the influence of SPOP's promotion of CDCA5 ubiquitination and degradation on the growth of Pca cells. This study provides further molecular insights into its tumor suppressor effect in the development of PCa.

Materials and methods

Antibodies and reagents

Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, New York). The antibodies used in this study are as follows: CDCA5 (ab192237, 1:1,000 WB, abcam, Cambridge, UK); SPOP (16750-1-AP, 1:1,000 WB, Proteintech, Wuhan, China); Cyclin A (sc-751, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), Cyclin B1 (4138s, 1:1,000 WB, Cell Signaling, Beverly, MA); Cyclin D (55506S, 1:1,000 WB, Cell Signaling); AKT (10176-2-AP, 1:1,000 WB, Proteintech, Wuhan, China); p-AKT (Ser473)(66444-1-Ig, 1:1,000 WB, Proteintech, Wuhan, China); GSK3β(22104-1-AP, 1:1,000 WB, Proteintech, Wuhan, China); p-GSK3β(Ser9) (D3A4, 1:1,000 WB, Cell Signaling, Beverly, MA); Bcl-2 (D55G8) (4223, 1:1,000 WB, Cell Signaling, Beverly, MA); Bax (D2E11) (5023, 1:1,000 WB, Cell Signaling, Beverly, MA); Cleaved Caspase-3 (Asp175) (5A1E) (9664, 1:1,000 WB, Cell Signaling, Beverly, MA); Cleaved Caspase-9 (Asp315) (D8I9E) (20750, 1:1,000 WB, Cell Signaling, Beverly, MA); Cleaved PARP (Asp214) (D64E10) (5625, 1:1,000 WB, Cell Signaling, Beverly, MA); EdU Apollo 488 kit (C10310-3, RIB-BIO, Guangzhou, China). GAPDH (60004-1-1g, 1:5000 WB, Proteintech); Tubulin (66240-1-1g, 1:5,000 WB, Proteintech); VINCULIN (66305-1-1 g, 1:5,000 WB, Proteintech). Secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology. The secondary antibodies for immunofluorescence staining are Alexa fluor 488, 594, 647 antimouse, -goat or -rabbit from Jackson Immuno Research Laboratories (West Grove, PA).

Short interfering (si)RNA was synthesized by GenePharma (Suzhou, China): si Negative control (NC), UUCUCCGAACUGUCACGU; siCDCA5-1, GCAGUUUGAUCUCCUGGUUU; siCDCA5-2, GCCAG GACUUGGAAAUGUUU; siSPOP-1, CAACUAUCAUGCUUCGGAU; siSPOP-2, AAAUGGUGUUUGCGAGUAA.

The sequences of short hairpin (sh)RNA were synthesized according to the oligonucleotides encoding the above indicated siRNA and then cloned into the plko.1 vector (Sigma Aldrich, St. Louis, MO, USA). For CDCA5 and SPOP expression, the cDNA (wild type or mutant) was cloned into the vector pHage, pCMV, or pIRES.

Cell culture and transfection

HEK293T (293T), PC3, Du145, and HeLa cells were obtained from ATCC. The HeLa-Fucci cell line is a gift from Dr. Daniel Durocher from the University of Toronto. The cells mentioned above were cultured in F12K or DMEM supplemented with 10% FBS and 1% Pen-Strep (100 units/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere with 5% CO_2.

RNAiMAX or Lipofectamine 3000 (Life Technology) was applied to transfect the cells with siRNA or cDNA according to the manufacturer's protocols. A 48 to 72 h after transfection, the cells were used for further analyses.

The plasmids used in this work were produced by standard cloning methods. For lentivirus packaging, the plasmids were co-transfected into 293T cells with the 2 helper plasmids psPAX2 and pMD2G (Addgene). A 48 h after transfection, the supernatant containing the lentivirus was collected and filtered through a 0.45-µm-mesh filter. Lentiviruses with overexpression or knockdown constructs were produced in the laboratory and used to infect the above cell lines with 2-d puromycin treatment (Sigma Aldrich; 2µg/mL) to select the infected cells.

Sample preparation and LC-MS/MS analysis

Cells were lysed in a lysis buffer (10% DOC, 1mM Tris, pH8.8) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). Lysate was centrifuged at 16,000 g for 10 min at 4°C. The supernatant was digested with sequencing-grade trypsin, and then the resulting peptide was separated into 9 fractions, and then analyzed by LC-MS/MS. The MS data was processed on the Firmiana platform. The FDR of peptide and protein levels estimated by searching the bait database were both 1%. The gene symbol has been updated to GENCODE.vM23 (09/19/2019). The protein is quantified by the label-free, intensity-based absolute quantification (iBAQ) method and further standardized to iFOT (intensity-based ratio multiplied by 10^5).

Western blot (WB) analysis

Cells were harvested after the indicated treatments and lysed with RIPA buffer (Applygen Technologies Inc., Beijing, China), supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany). Proteins were loaded on SDS-PAGE gel and transferred to a nitrocellulose filter membrane (NC, PALL, New York), and detected with the indicated antibodies.

Cell synchronization and FACS analysis

HeLa cells at 20% confluency was cultured in DMEM containing 2 mM thymidine for 18 h, released to fresh DMEM for 9 h, then cultured in DMEM containing 2 mM thymidine for 18 h. Cells were synchronized in late G1-phase and then collected at the indicated time points.

Cell cycle and apoptosis

Cells were fixed and permeabilized in 70% ethanol overnight. The samples were digested with 25 µg/mL RNase and stained with 40 µg/mL propidium iodide (PI) for 30 min at 25°C. BD's apoptosis kit was applied to the apoptosis experiment. The cells were sorted with BD FACS Canto II Flow Cytometer. The results were analyzed by ModFit LT 4.1 and Flowjo.

Ubiquitination assay

DU145 or 293T cells were first infected siSPOP, CDCA5, SPOP, or related mutant-type cDNA as indicated, replaced with fresh medium after 12 h, and then transfected with HA-Ub plasmid again. The collected cells were lysed in 100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 6 M Urea and 0.5% SDS (lysis buffer) plus protease inhibitors cocktail (Roche) after being treated with MG132 (10 μM) for 8 h. After sonication, the supernatants were incubated with anti-Flag M2 affinity beads (Sigma) for 6 h at 4°C. The beads were then spun down, washed with the NETN lysis buffer, and prepared for WB analysis.

Quantitative RT-PCR

Trizol reagent (Invitrogen) was used to isolate total RNA from cells. One microgram of RNA extract was then reverse transcribed using RT reagent kit (Takara). FastStart Universal SYBR Green master mix (Roche) was used for quantitative PCR. The relative expression levels of target gene mRNA were normalized to the expression levels of GAPDH.

ImmunoFluorescence (IF)

Cells after indicated treatment were plated on coverslips, fixed with 4% paraformaldehyde for 15 min, permeabilized in PBS containing 0.5% Triton X-100 for 5 min, and blocked with 5% BSA in PBS for 1 h at room temperature, followed by incubation with primary antibodies at 4°C overnight. After 3 washes in PBS, the coverslips were incubated with secondary antibodies for 20 min at 37°C. All images were taken on a Nikon Ni-E microscope (Nikon Corporation, Tokyo, Japan), with identical exposure times for each sample.

MTS analysis for cell proliferation

After lentivirus infection, the PCa or DU145 cells were trypsinized, seeded in a 96-well plate (1.5×10^3 cells/well), and grown at 37°C. At the end of the designated incubation time, cell proliferation was assessed by using MTS colorimetric analysis (Promega, Madison, Wisconsin, USA). After removing the medium, 20 μL MTS plus100 μL complete medium was added into each well, and then the cells were incubated () for 1 h. The absorbance of the formazan product was recorded at 490 nm using a Spectra MAX 190 microplate reader (Molecular Devices, CA).

Colony formation assays

After lentivirus infection and puromycin treatment for 2 d, trypsinized PCa cells were seeded into a 6-well plate (1,000 cells/well). After 10 d (when colonies were formed), the plate was washed with PBS, and the cells were fixed with 4% paraformaldehyde (PFA). After 15 min, the cells were stained with crystal violet for 10 min. After washing 3 times with PBS, the stained colonies were observed and photographed.

Chromosome spreading

Cells treated with colchicine (100 ng/mL) for 4 h were harvested with trypsin, and resuspended in 0.075M KCl for 25 min at 37°C. The cells were pelleted by centrifugation at $1000 \times$ g for 5 min, gently resuspended in a small amount of residual hypotonic medium, and fixed by ice-cold fixative (methanol: acetic acid; 3: 1). The smear was prepared by dropping the cell
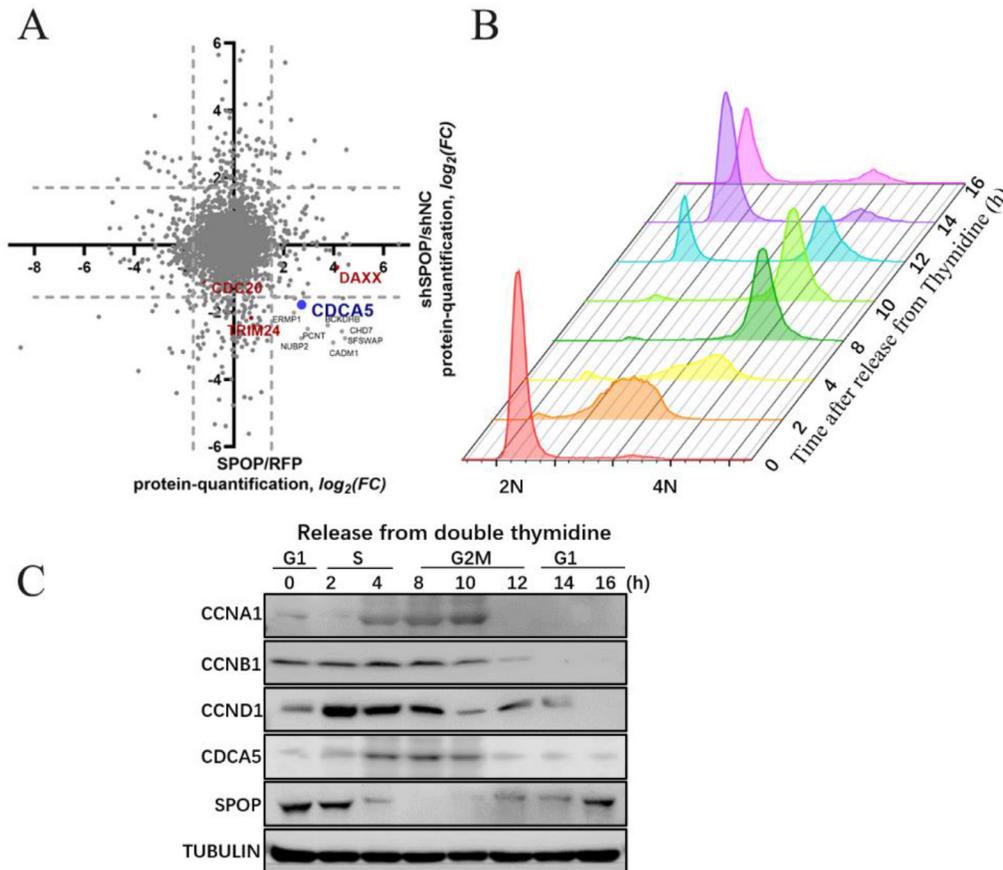

Fig. 1. The protein abundance of CDCA5 and SPOP tended to be opposite and fluctuated during cell cycle progression
(A) 293T cells were stably infected with the corresponding shRNA or cDNA after 1-week puromysin (puro) screening, then the protein profiling was detected. Scatter plot of normalized protein level changes between shSPOP vs shNC (depicted as log2 ratio of shSPOP /shNC) and SPOP vs RFP (depicted as log2 ratio of SPOP /RFP) shows corresponding protein expression changes.
(B, C) Westernblot (WB) analysis of protein derived from Hela cells at different cell cycles(cells were synchronized at the G1/S boundary then released back into the cell cycle and collected at the indicated points in time). The cell-cycle profiles in D were detected by fluorescence-activated cell sorting (FACS) and analysed by ModFit LT.
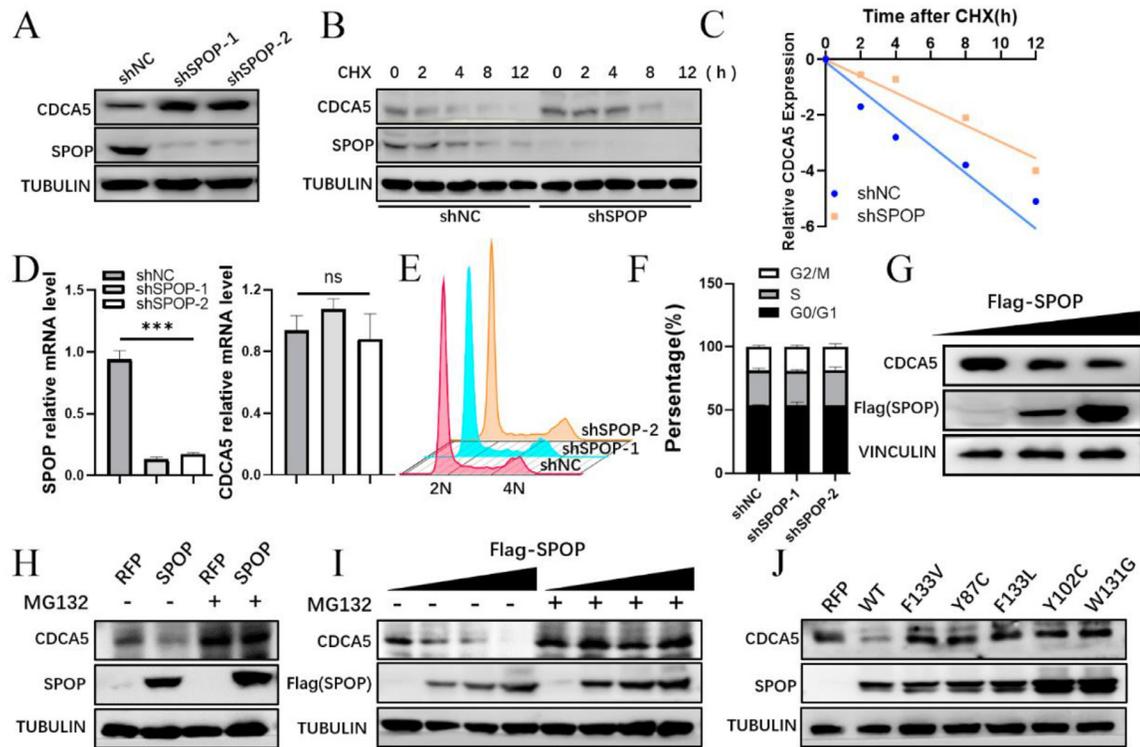
Fig. 2. Wild-type SPOP, not SPOP mutant, negatively regulates the stability of CDCA5 protein

(A) DU145 cells were infected with shSPOP lentiviral, 2 mg/mL puromycin was used to filter the uninfected cells for 72 h. The samples were collected and detected by WB

(B) DU145 cells infected with indicated constructs were treated with 100 mg/ml CHX for different time courses. Samples were tested by WB.

(C) Quantification of WB shown in B using Image J software. CDCA5 immunoblot bands were normalized to Tubulin, then normalized to the t = 0 time point.

(D-F) The infected cells were collected and detected by qPCR (D, ***P <0.001, ^{ns} P > 0.05), and cell cycle (E, F) Data are mean ± SEM, P >0.05).

(G) WB analysis of WCL derived from 293T cells transfected with the indicated constructs.

(H-I) 293T cells were transfected with the indicated constructs, cells were pretreated with 10 mM MG132 for 8 h before harvesting.

(J) 293T cells were transfected with SPOP WT and PCa-related mutants to detect the corresponding protein by WB.

suspension onto a cold clean glass slide and allowed to air dry. The smear was stained with Giemsa (Karyomax, Invitrogen) according to the manufacturer's instructions. Images were taken on a Nikon Ni-E microscope (Nikon, Tokyo, Japan).

Statistical analysis

For statistical analysis, Student's t test was used for parametric variables. All experiments were performed at least 3 times, and P < 0.05 was considered statistically significant.

Results

Proteomic profiling identifies CDCA5 as a potential SPOP substrate

To analyze SPOP-dependent proteomic change, we first performed the whole proteome profiling in SPOP knockdown or overexpression cells. Five hundred and fifty-six proteins exhibited a more than 2-fold increase in shSPOP compared with shNC, including known substrates DAXX, TRIM24. Two hundred and thirty-eight proteins showed a more than 2-fold decrease in overexpressed SPOP compared with the control RFP, including TRIM24 and CDC20. As shown in Fig. 1A, the lower right quadrant marks

the proteins whose change exceeded 3-fold, including CDCA5, ERMP1, BCKDHB, and other previously unidentified potential substrates. Since SPOP is the substrate recognition protein of an E3 ubiquitin ligase, its main function is to mediate the degradation of substrate ubiquitination. Therefore, we compared the above proteomics data with the SPOP ubiquitin mass spectrometry data reported by the Janouskova group [22], and looked for potential substrate proteins whose ubiquitination was increased. Among them, we selected CDCA5 for subsequent confirmation (Fig. 2C, Fig. 3A, B). As mentioned above, CDCA5 is overexpressed in many types of cancers [18-21]. Meanwhile, CDCA5 may be a poor prognostic factor in patients with PCa (Fig. 7E-G). The expression and the role of CDCA5 in PCa still need to be further explored.

Since the mass spectrometry results showed that there was a negative correlation between the expression level of SPOP and CDCA5, we investigated the cell cycle dependence of SPOP and CDCA5. We synchronized the cell cycle to G1/S by double thymidine block and collected the cells at various times after release (Fig. 1B, C). The protein levels of SPOP and CDCA5 both fluctuated with the cell cycle. CDCA5 protein abundance was high in late S and G2, peaked in early M phase, followed by a sharp reduction in late M/G1 phases. In contrast, SPOP protein abundance was high during G1 phase, followed by a sharp reduction in S phase. These trends were consistent with the MS data, and supported the hypothesis that SPOP may regulate the CDCA5 protein stability.
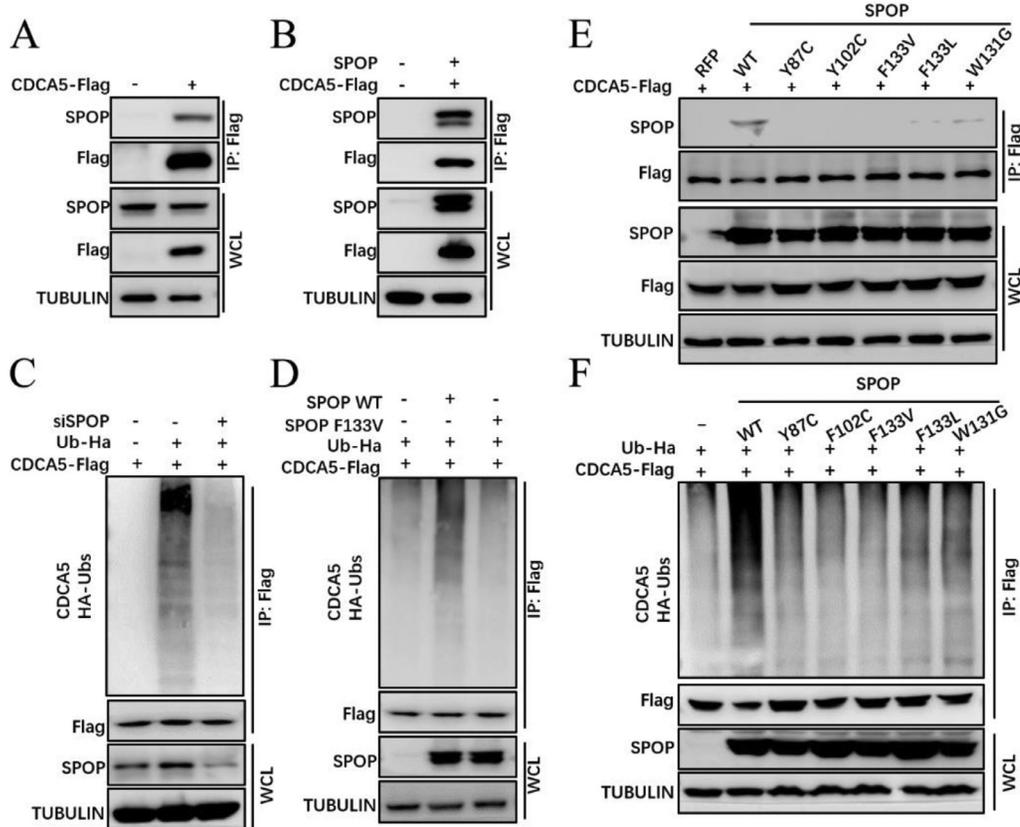
Fig. 3. Wild-type SPOP, not SPOP mutant, can interact with CDCA5 to promote its polyubiquitination degradation
(A- B, E) CO-Immunoprecipitation (IP) from 293T cells with the indicated constructs. After 48 h of transfection, cells were pretreated with 10 mM MG132 for 8 h before harvesting.
(C-D, F) WB of WCL and flag IP of DU145 cells transfected with the indicated constructs. Cells were treated with 10 mM MG132 for 8 h and lysed with denature buffer.

Wild-type SPOP, not the PCa-associated SPOP mutant, negatively regulate the CDCA5 protein stability

The above data showed that there is a negative correlation between SPOP and CDCA5 protein levels. We next determined whether there was a regulatory relationship between the 2 at the transcriptional or post-translational modification levels. We found that proteasome inhibitor MG132 could stabilize the endogenous CDCA5 levels in PCa cell lines DU145 and PC3 (Fig. S1A). We used protein synthesis inhibitor cycloheximide (CHX) to measure CDCA5 half-life. MG132 treatment led to a prolonged half-life of endogenous CDCA5 in DU145 cells (S1B, C). These results suggest that CDCA5 was degraded through the proteasome pathway.

Next, we knocked down SPOP by shRNA in DU145 cells and found that the endogenous CDCA5 protein level was increased (Fig. 2A), and the half-life was prolonged (Fig. 2B-C), but its mRNA expression was not significantly changed (Fig. 2D). Since our previous results showed that CDCA5 protein expression levels fluctuated, it is possible that loss of SPOP affected the cell cycle and thus the protein expression of CDCA5. To test this hypothesis, we measured the cell cycle progression after knocking down SPOP in the DU145 cell line, and found no significant cell cycle changes (Fig. 2E, F). This conclusion was also verified in HeLa-Fucci cells (Fluorescent Ubiquitination-based Cell Cycle Indicator: G1 phase, CDT1-RFP showed red fluorescence in the nucleus. S, G2, and M phases, Geminin-GFP showed green fluorescence. During the G1/S transition, Cdt1 reduced and Geminin increased, leading

to yellow fluorescence). As shown in Supplemental Fig. 1D - G, the CDCA5 protein level was increased, and the ratio of cell cycles was not significantly different in SPOP silencing cells.

Next, we overexpressed SPOP in 293T cells and found that the CDCA5 protein level was reduced in a dose-dependent manner (Fig. 2G). In addition, SPOP-dependent CDCA5 degradation could be blocked by the proteasome inhibitor MG132 (Fig. 2H, I), indicating that SPOP mediated CDCA5 degradation through the 26S proteasome pathway. In the molecular classification of PCa, SPOP mutation ranks second among all gene mutations. The most frequent SPOP mutations associated with PCa are F133V, F133L, Y87C, F102C and W131G. Once these sites are mutated, the binding ability of SPOP to substrates will be disrupted, thereby affecting the ubiquitination and degradation of downstream substrates [1]. Compared to wild type SPOP(SPOP WT), the above PCa derived SPOP mutants were incapable of promoting CDCA5 degradation (Fig. 2J).

Since SPOP is the scaffold protein in the E3 ubiquitin ligase complex that can specifically recognize and promote substrate ubiquitination and degradation [2], we further examined whether SPOP can specifically bind CDCA5 and promote its poly-ubiquitination and degradation. We found that CDCA5 interacted with SPOP (Fig. 3A, B). Consistently, knockdown SPOP led to reduced CDCA5 poly-ubiquitination level in cells (Fig. 3C). While the ubiquitination level of CDCA5 was greatly increased by SPOP WT (Fig. 3D), SPOP carrying PCa associated mutations failed to interact with CDCA5 to promote its polyubiquitination and degradation (Fig. 3E, F).
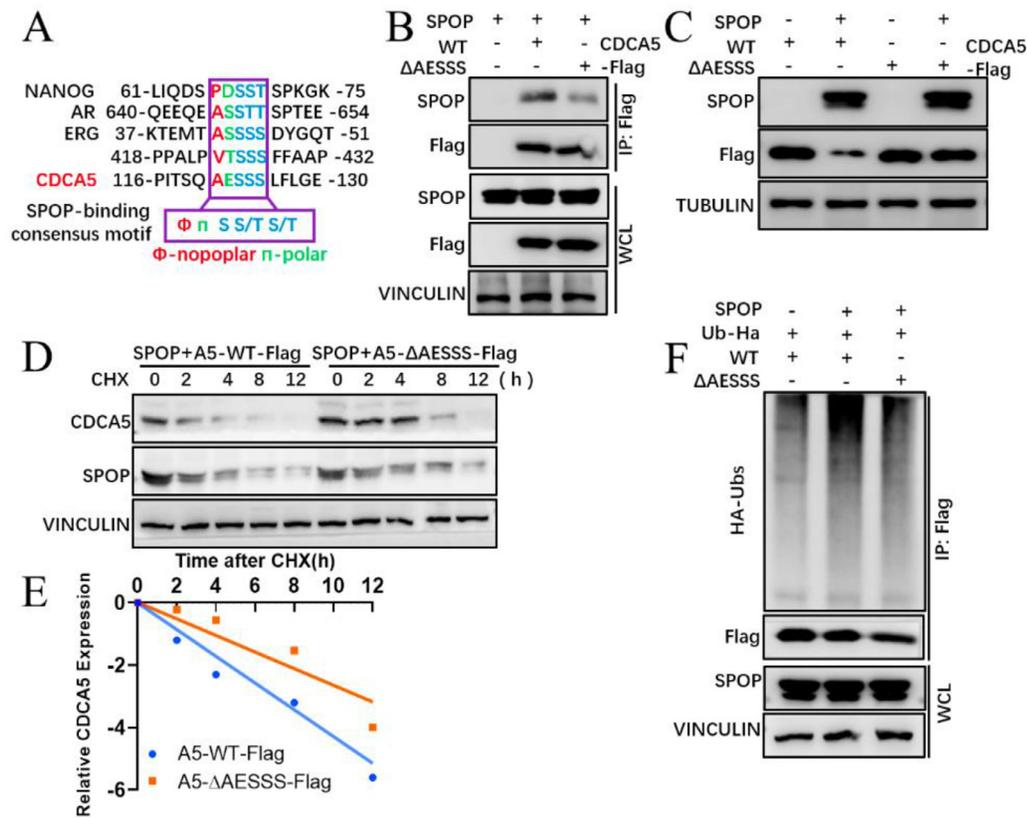
Fig. 4. The $_{121}$AESSS$_{127}$ motif in CDCA5 is a degradation determinant recognized by SPOP
(A) The potential SPOP binding motif in CDCA5 is compared with the sequence of known SPOP substrates.
(B) 293T cells were transfected with indicated constructs, WB analysis of WCL and immunoprecipitation (IP).
(C) SPOP was overexpressed in 293T cells overexpressing CDCA5 WT or deletion mutant (ΔAESSS), and related protein expression was detected by WB.
(D) 293T cells transfected with indicated constructs were treated with 100 mg/mL CHX for different time course. WCL was prepared and western blotted.
(E) Quantification of WB shown in D using ImageJ software. CDCA5 immunoblot bands were normalized to Vinculin, then normalized to the t = 0 time point.
(F) WB of WCL and flag IP of DU145 cells transfected with the indicated constructs. Cells were treated with 10 mM MG132 for 8 h and lysed with denature buffer.

To find SPOP substrate recognition motif, we compared the sequence of several established SPOP substrates, including AR [5] and ERG [6], NANOG [8], and found that they all share a common SPOP-binding consensus motif Φ-π- S-S/T-S/T (Φ: non-polar amino terminal; π: polar amino acid; S: serine; T: threonine). Sequence analysis identified a motif $_{121}$AESSS$_{127}$ located at the N-terminus of CDCA5 (Fig. 4A). Deleting this putative binding motif (CDCA5-ΔAESSS) impaired CDCA5 interaction with SPOP (Fig. 4B), and consequently, the CDCA5-ΔAESSS mutant became resistant to SPOP-mediated destruction (Fig. 4C-4E) and showed a reduced poly-ubiquitination (Fig. 4F). Taken together, these results demonstrate that the $_{121}$AESSS$_{127}$ motif is required for CDCA5 interaction with SPOP, and the subsequent poly-ubiquitination and degradation.

SPOP regulates CDCA5 expression and influences PCa cells proliferation as well as apoptosis via the AKT pathway

We next examined the effect of CDCA5 depletion on cell proliferation. CDCA5 depletion led to a significant decrease in cell growth, and a reduction in cloning ability (Fig. 5A, B, S2A-C). Consistent with these results, we found that stable depleting of CDCA5 caused G2M phase arrest and a significant increase of apoptosis (Fig. 5C, D, S2C-E). Since the main function of CDCA5 is regulating the aggregation and separation of sister chromatids, we then performed chromosome spreading analysis. CDCA5 depleted cells displayed severe disruption of chromosomal function: the chromosome karyotype was seriously disordered, and the proportion of paired chromosomes was decreased (Fig. 5E). On the other hand, in CDCA5 overexpressed cells, the proliferation speed was accelerated (S2G). Together, these data suggest that loss of CDCA5 makes the cell unable to accumulate the sister chromatids correctly, leading to growth arrest in G2M phase, and cell apoptosis.

To explore the underlying mechanism regulated by CDCA5, we analyzed a series of apoptosis-associated pathways by WB. The results showed that p-AKT (Ser473) and p-GSK3β were decreased in CDCA5-knockdown cells. WB analysis also showed that CDCA5 depleting increased Bax/Bcl-2 ratio and increased expression of cleaved PARP and Caspase-3/9 (Fig. 5F, S2G).

The above results indicate that CDCA5 plays a critical role in maintaining growth of PCa cells via AKT pathway. Next, we examined the effect of SPOP on cell proliferation by promoting the degradation of CDCA5. DU145 and PC3 colony formation ability was enhanced when SPOP expression was silenced with 2 different shRNA (Fig. 6A, B). Compared with shNC and simply SPOP knockdown cells, knocking down CDCA5 on the basis of knocking down SPOP leads to reduced cell proliferation. In the meanwhile, compared with overexpression of CDCA5 alone, overexpression of both SPOP and CDCA5 reduced cell proliferation rate (Fig. 6C-F), suggesting
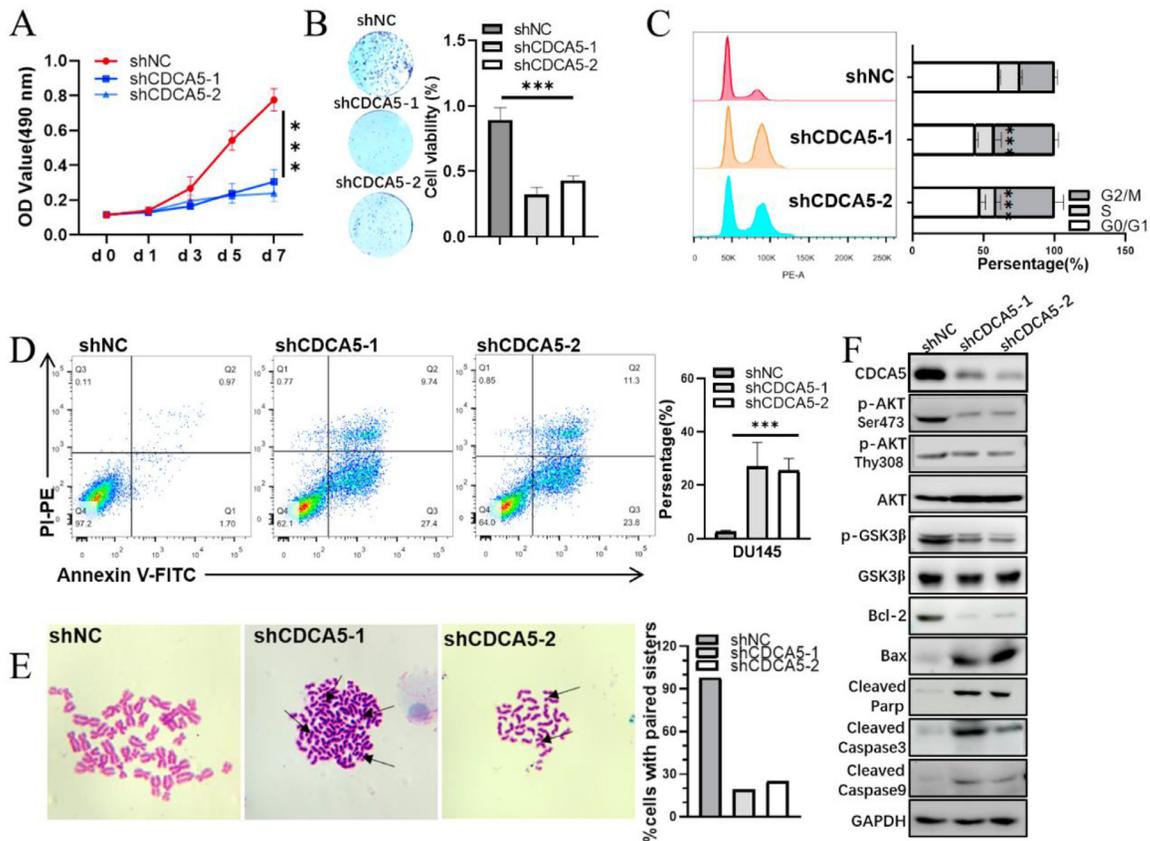
Fig. 5. CDCA5 is necessary to maintain the growth of DU145 cells

(A-E) Growth curve of the DU145 cells with the indicated constructs (A, ***$P < 0.001$), clony Formation (B, ***$P < 0.001$), cell cycle (C, ***$P < 0.001$), cell apoptosis (D, the cells in the upper right quadrant indicate the occurrence of mid-late apoptosis, and the lower right quadrant indicates the occurrence of early apoptosis. F counts the overall apoptosis rate, Data are mean ± SEM, ***$P < 0.001$) and chromosome karyotype analysis (E, a stable CDCA5 depleting cell line were cultured for about one week. CDCA5 depleting cells showed obvious sister chromatid aggregation defects, and weak physical connections between sister chromatids could be observed occasionally (see Arrow, $n \geq 50$). F: WB analysis of Protein levels of AKT, GSK3β, Bcl-2, BAX, PARP, Caspase-3, and Caspase-9 in DU145 cells after transduction with shCDCA5 and shNC lentivirus.

that SPOP overexpression partially rescued the CDC5A overexpression phenotype. The above experiments have proved that SPOP can inhibit the proliferation and promote apoptosis of tumor cells by or partially inhibiting the expression of CDCA5 protein via AKT pathway (Fig. 6F).

Analysis of the CDCA5 in clinical PCa samples

Having established that SPOP-controlled CDCA5 expression regulates PCa cells proliferation in cell culture, we next analyzed the CDCA5 expression in clinical PCa samples. we analyzed the TCGA database and found that, the CDCA5 gene expression level was relatively higher in human PCa samples, while SPOP was lower, compared to normal prostate tissue (www.tnmplot.com, Fig. 7A, C, $P < 0.001$) [23].PcaPcaWe compared the expression of CDCA5 on the ULCAN platform (ualcan.path.uab.edu) [24] and found that its high expression predicted more severe lymph node metastasis, and higher Gleason score (Fig. 7B, C), which suggested a high degree of correlation between CDCA5 expression and PCa development. Consistent with high expression at the gene level, the protein level of CDCA5 was also upregulated in PCa tissues (ATLA provisional dataset [25,26], Fig. 7D). Importantly, higher CDCA5 expression was significantly correlated with poor overall survival and disease-free survival of patients with PCa (gepia.cancer-pku.cn [27], Fig. 7D-F, Log rank $P < 0.05$).

Discussion

PCa is a common malignant that is ranked second among all malignant tumors in men worldwide and remains one of the diseases with a high mortality rate. In 2020, there were more than 1.41 million new cases and more than 370,000 deaths worldwide [28,29]. PCa is a complex and heterogeneous disease, and different subtypes respond differently to clinical treatment methods [30, 31]. At the molecular level, *SPOP* single gene mutation is one of the common types of genetic alteration in PCa, accounting for about 11% of all mutations [2,32]. Although a large number of studies [5,33-35] have reported the important roles that SPOP plays in tumors of many different cancer subtypes including PCa, finding more SPOP substrates and regulators could help understand the underlying mechanism and provide useful resources for cancer research.

In this study, we found that SPOP, an E3 ubiquitinated ligase complex, can regulate the protein stability of a oncoprotein CDCA5 by promoting its ubiquitination and degradation. The SPOP proteins with mutations in the MATH domain, such as F133V, Y87C, F102C, and W131G, showed a greatly reduced ability to regulate CDCA5, which may partially explain the overexpression of CDCA5 in PCa. Our results showed that, when CDCA5 was overexpressed in DU145 cell line, the chromosome karyotype was largely normal, and the proliferation was significantly increased. As tumor cells have
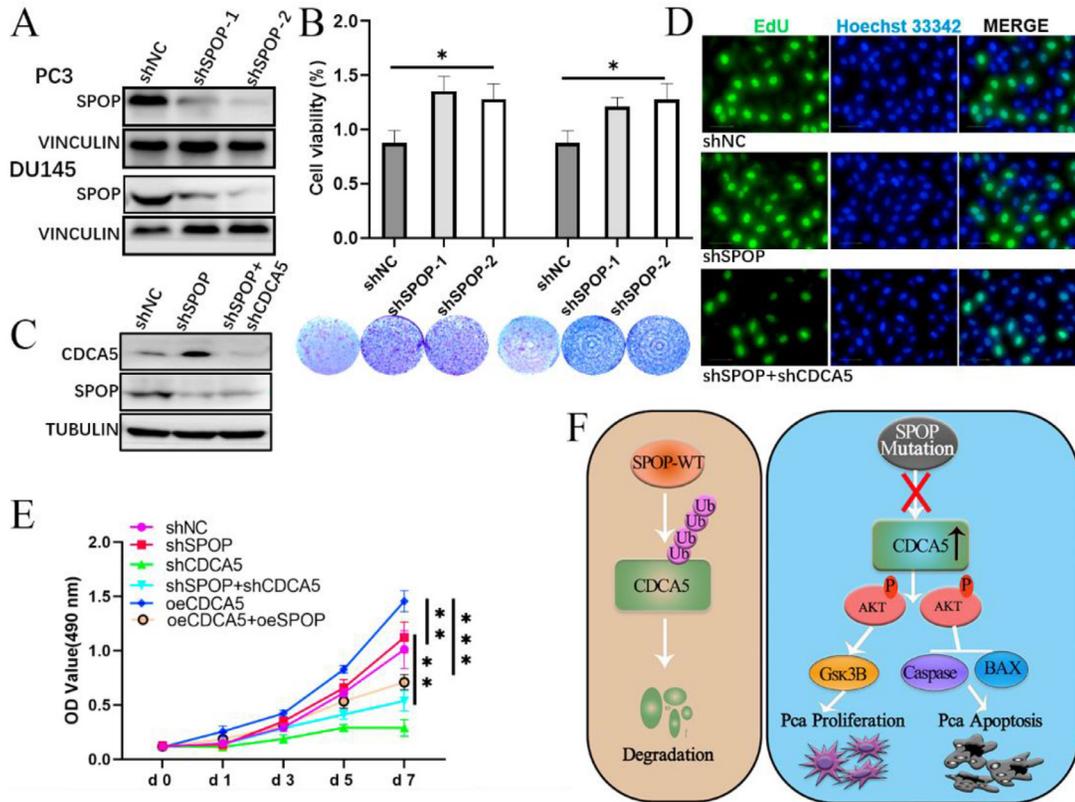
Fig. 6. SPOP affects the growth of PCa cell lines through or partially through CDCA5

(A) DU145 and PC3 cells were infected with SPOP lentiviral shRNA, 1 mg/mL puromycin was screened for 72 h, and the knockdown efficiency was detected by WB;

(B) the cloning ability of the 2 cell lines (*$P < 0.05$).

(C, D) DU145 cell line infected with SPOP lentiviral shRNA, 2 mg/mL puromycin was screened for 72 h, then shCDCA5 and shNC lentiviruswere added again, 48 h later, WB was used to detect knock-down efficiency and Edu to detect proliferation, green fluorescence EdU staining positive cells, blue is the nucleus stained by Hoechst33342;

(E) DU145 cell lines were processed according to the corresponding treatment methods, and the 7-d growth curve (F, Data are mean $\pm$ SEM, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) on the 96-well plate was detected.

(F) Schematic of the proposed mechanism model of SPOP-WT promotes oncoprotein CDCA5 subsequent ubiquitination and degradation in PCa. Therefore, SPOP mutation leads to elevated CDCA5 protein levels by evading the SPOP-mediated degradation pathway to promote PCa progression.

indefinite proliferating ability, and their cell division interval is significantly shortened [17], it suggests that cell division proteins, like CDCA5, may need to be maintained at high levels to support the continuous division activities.

CDCA5 is essential for the cells to survive. Loss of CDCA5 can cause miss-assembly of chromatids. The sister chromatids are separated in an immature state, and the cells fail to enter metaphase [16, 36, 37]. Consistent with previous reports, we also found that, upon CDCA5 knocking down, the ratio of paired sister chromatid was greatly reduced, and the distance between the 2 chromatids was also greatly increased, resulting in severe G2M phase block and apoptosis. CDCA5 silencing has been found to induces suppression of AKT signaling pathway in Hepatocellular Carcinoma [18]. AKT-related signalling pathway plays a key role in numerous cancer processes, such as promote cell proliferation, and inhibit apoptosis. Phosphorylated AKT can affect proliferation by regulating the activity of the cyclin D1 kinase glycogen synthase kinase-3β (GSK3β) [38,39]. AKT also directly phosphorylates several components, such as Bcl-2, Bax, and pro-death protease caspase-3 and caspase-9 [40–44]. We found CDCA5 knockdown reduced the level of AKT phosphorylation at Ser473 site. This leads to a decrease in the expression of

proliferation-related proteins and an increase in the expression of apoptosis-related proteins, which in turn causes the growth of PCa cells to stagnate and increase apoptosis.

Previous studies showed that APC^{CDH1} is an E3 Ub ligase that regulates CDCA5 stability during G1 [16]. We show here that SPOP, like CDH1, can also promote the ubiquitination and degradation of CDCA5. It is not clear how these 2 E3 ubiquitination ligases coordinate in regulating CDCA5 degradation, for instance, whether SPOP and CDH1 function in a temporal and spatial manner to achieve accurate control CDCA5 stability. Future studies are needed to fully understand this regulation.

Our data showed that SPOP may regulate PCa cell growth by modulating the polyubiquitin degradation of the oncoprotein CDCA5. Consistent with this notion, CDCA5 is highly expressed in PCa tissues, and correlates with worse clinical stage, pathological grade and poor prognosis. The low expression of SPOP or PCa-associated SPOP mutations in PCa, impair its ability to regulate downstream target protein, such as CDCA5, and the level of the oncoprotein CDCA5 is further abnormally high. With the continuous maturity of individual genetic testing technology, more and more patients can benefit from personalized diagnosis and targeted therapies.
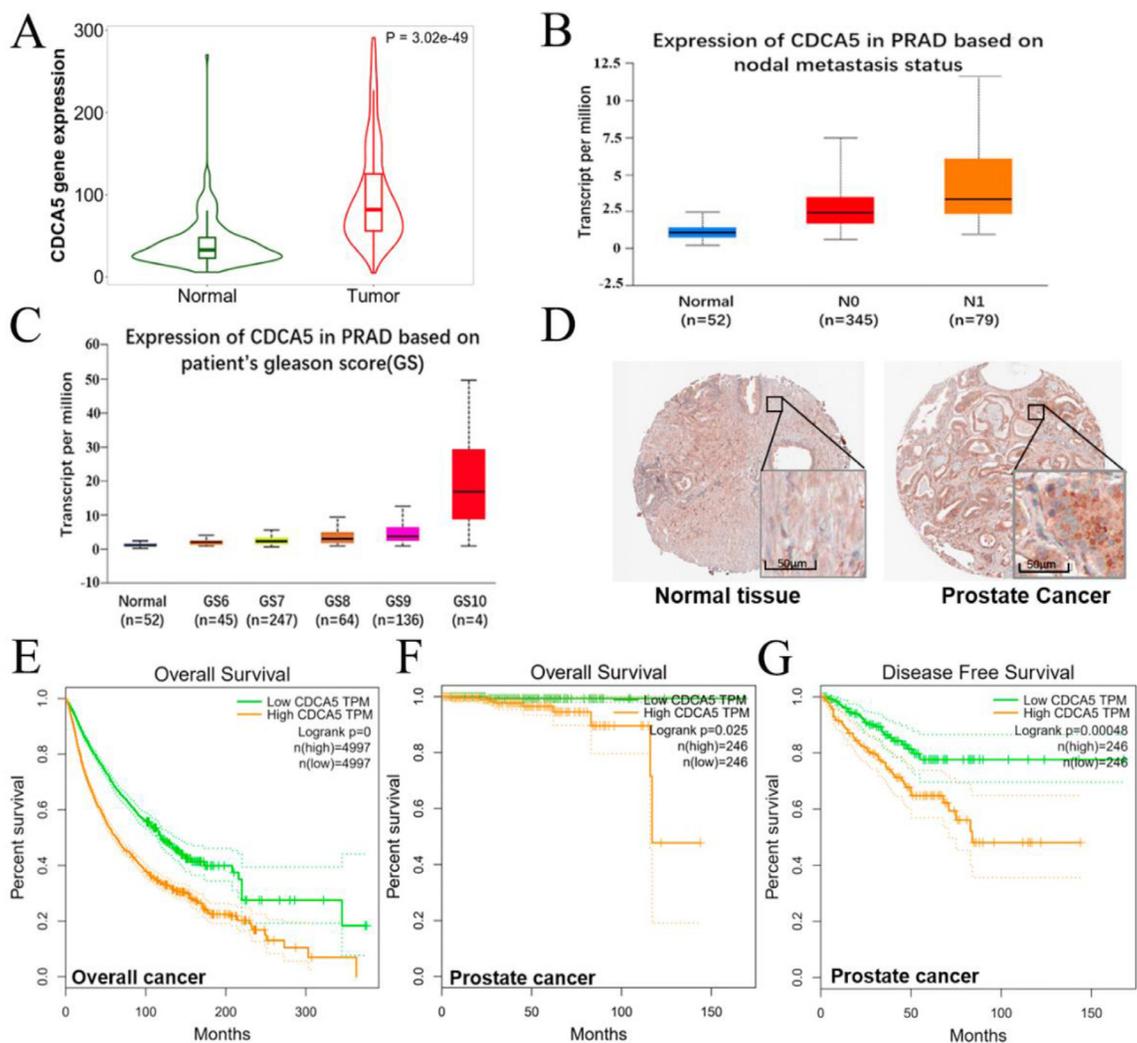
Fig. 7. CDCA5 is significantly high expressed in PCa samples, and its high expression indicates a poor prognosis.
PcaPCa (A) The gene expression of CDCA5 in human PCa samples compared with normal samples from TCGA database.
(B-C) The expression of CDCA5 in PCa tissue is positively correlated with the status of lymph node metastasis and the patient's Gleason score.
(D) CDCA5 protein is highly expressed in PCa tissues compared with normal samples.
(E-G) Survival analysis of CDCA5 in overall cancer and Pca patients.

Conflict of interest

The authors have declared that no conflict of interest exists.

Author contributions

Zhenzhen luo: Conceptualization, Methodology, Investigation, and Writing; Jing Wang, Yue Zhu: Protein profiling experiment and Software; Xiao Sun, ChenChen He, Mengjiao Cai: Review & Editing. Jinlu Ma: Commentary or Revision. Yi Wang: Writing- Reviewing and Editing; SuXia han: Supervision, Reviewing and Editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2021.08.002.

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